Video Article

Intravital Microscopy of Leukocyte-endothelial and Platelet-leukocyte Interactions in Mesenterial Veins in Mice

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Abstract

Intravital microscopy is a method that can be used to investigate different processes in different regions and vessels in living animals. In this protocol, we describe intravital microscopy of mesentery veins. This can be performed in a short period of time with reproducible results showing leukocyte-endothelial interactions *in vivo*. We describe an inflammatory setting after LPS challenge of the endothelium. But in this model one can apply many different types of inflammatory conditions, like bacterial, chemical or biological and investigate the administration of drugs and their direct effects on the living animal and its impact on leukocyte recruitment. This protocol has been applied successfully to a number of different treatments of mice and their effects on inflammatory response in vessels. Herein, we describe the visualization of leukocytes and platelets by fluorescently labeling these with rhodamine 6G. Additionally, any specific imaging can be performed using targeted fluorescently labeled molecules.

Video Link

The video component of this article can be found at http://www.jove.com/video/53077/

Introduction

The purpose of this protocol is to describe a simplified technique of intravital microscopy of mesentery veins in living mice for direct observation of leukocyte-endothelial and leukocyte-platelet interactions under inflammatory conditions.

Intravital microscopy was developed to study leukocyte-endothelial interactions *in vivo* under inflammatory conditions[1,2], which is not trivial but important for understanding inflammatory leukocyte recruitment and function. The method we describe in this protocol was developed based on previously published publications. Similar to visualizing platelet incorporation in a growing thrombus[3] and in parallel to what has been published earlier[4], an exteriorized mesentery vein is examined by transmitted light microscopy. There are various other models of intra-vital microscopy such as the cremaster muscle of rats[5] or liver of mouse and rats[6].

Intravital microscopy of mesentery vein has been developed and applied in previous studies by several groups. We have used this technique to observe differences in leukocyte recruitment between wild type mice and tryptophane hydroxylase1 (Tph1) -/- mice, which are deficient of non-neuronal serotonin and compared the findings to mice whose platelet serotonin was pharmacologically depleted by long-term application the a selective serotonin reuptake inhibitor fluoxetine[7]. We have also examined leukocyte-endothelial interactions after acute fluoxetine treatment[8].

In this protocol we focus on intravital microscopy of mesentery veins, because this model can be performed quickly, and allows valid measurements of leukocyte-endothelial and platelet-leukocyte interactions. This is much more challenging and time-consuming in intravital microscopy of other organs, such as bone, liver, skin, or cremaster muscle. The model described here is ideal for reproducible evaluation of inflammatory cell-cell interaction after challengeing it with an inflammatory stimulus, such as intraperitoneal injection of lipopolysaccharide.

Protocol

All animal experiments were performed in compliance with the German animal protection law (TierSchG). The mice were housed and handled in accordance with good animal practice as defined by FELASA (www.felasa.eu/guidelines.php) and the national animal welfare body GV-SOLAS (www.gv-solas.de/index.html). The animal welfare committee of the University of Freiburg as well as the local authorities (Regierungspräsidium Freiburg) approved all animal experiments.

1. Animal Handling, Preparation and Induction of Inflammation

1. Use 4 - 6 week old male mice with a weight range of 16 - 20 g. Note: If the mice are older or heavier they present excessive fat surrounding the vessel, limiting the microscopic observation.
2. Sterilize all tools and microscope table prior to surgery.
3. Inject 20 mg/kg LPS (lipopolysaccharide) intraperitoneally 4 hr prior to microscopy into the living mouse to induce a bacterial inflammation.
4. Prewarm a 0.9% saline solution in a waterbath at 37 °C to humidify the plastic chamber and the mesentery tissue.
5. Anesthetize the mouse with intraperitoneal injection of ketamine (100 mg/kg) and xylazine (5 mg/kg) right before the microscopy procedure. Alternative anesthesia can be used, *e.g.,* 2% isoflurane inhalation. Confirm proper anesthetization by the loss of response to reflex stimulation (toe or tail pinch with firm pressure).
6. Depilate the abdomen using a shaver and remove loose hair with gauze saturated with ethanol 70%.
7. Apply vet ointment on the eyes of the mouse to prevent dryness, while under anesthesia.

2. Surgery

1. Sterilize the abdomen using 70% ethanol. This method is not a sterile method and could be lethal at the end of the experiment.
2. Perform a median laparotomy: Open the abdominal skin using small, curved forceps and small scissors. Identify the epigastrical vessels and open the peritoneum in the region of the linea alba, to protect the vessels.
3. Apply a few drops of prewarmed saline into the abdominal cavity to keep the tissue moist.
4. Label leukocytes and platelets fluorescently by injecting 50 µl rhodamine 6G (1 mg/ml) retro-orbitally as described before [9,10].
5. Exteriorize a loop of ileumand place it in a petridish with a diameter of 10 cm and make sure to keep the tissue moist by applying the 37 °C prewarmed saline solution (0.9%) every other minute.

3. Intravital Microscopy

1. Place the Mouse underneath the microscope and bring the mesentery vein with a diameter of 200 - 300 µm in the center of view. Choose a vessel with no visible fat surroundings.
2. Do not touch the mesentery vessels thereby avoiding stimulation of the endothelium. Handle the ileum loop cautiously.
3. Visualize blood cell-endothelial interactions with an inverted or upright microscope and a camera using a microscope software. Record blood cell-endothelial interactions for 1 min in 4 different veins per mouse.
4. Euthanize the mouse by cervical dislocation after the completion of imaging experiments.

4. Analysis

1. Carry out the analysis offline and blinded for all parameters. Carry out analysis using any suitable software program.
2. Confirm stable and interindividually comparable blood flow conditions in high time-resolution cine-clips (maximal frame rate) focused on intraluminal blood cell flow.
3. Quantify the number of rolling leukocytes. Therefor draw a vertical line through the vein and count all leukocytes crossing this line in 1 min.
4. Determine rolling velocity by measuring the time one single leukocyte needs to pass a distance of 50 µm while stably rolling on endothelium. To do this, draw two vertical lines in a distance of 50 µm through the vein.
    1. Measure the time a representative leukocyte needs to get from one line to the other.Calculate the speed of the leukocytes by dividing 50 µm through the time needed (µm/s).
5. Measure leukocyte adhesion in a field of 0.04 mm^2 . To do this, draw a square with the side length of 200 µm into the vein.
    1. Count the firm adherent leukocytes, defined as no visible movement for 30 sec, within this square.
6. Count the number of platelets bound to one leukocyte to quantify platelet-leukocyte interactions.

Representative Results

An overview of the experimental setting described in this protocol is shown in **Figure 1**. It shows a mouse with an exteriorized ileum-loop and its vessels, which can be observed in intravital microscopy. **Figure 2** shows certain degree of activation in untreated animals due to the procedure itself. But there is almost no slow rolling or firm adhesion of leukocytes compared to LPS-treated mice. **Figure 2** also shows the different results of intravital microscopy in wildtype mice either treated with fluoxetine for 3 weeks in the drinking water or untreated mice. **Figure 2A-C** show the results without any further inflammatory challenge with LPS, while mice in **Figure 2F-H** were treated with LPS in addition. Here we can see higher numbers of rolling and adherent leukocytes, while rolling velocity decreased after LPS challenge. With these experiments we could show that platelet serotonin is crucial for the initial steps of leukocyte recruitment in inflammation[7].

In **Figure 3,** intravital microscopy was performed without further LPS challenge and just after acute treatment with fluoxetine intraperitoneally 2 hr prior to surgery. Here we can see higher numbers of firmly adherent leukocytes and lower rolling velocities in fluoxetine treated mice, indicating an influence of acute fluoxetine treatment on leukocyte-endothelial interactions[8].

In **Figure 4** platelet-leukocyte interactions during lipopolysaccharide induced peritonitis are visualized within a vein. Platelets are rosetting around the leukocytes and these complexes are interacting with the vessel wall.

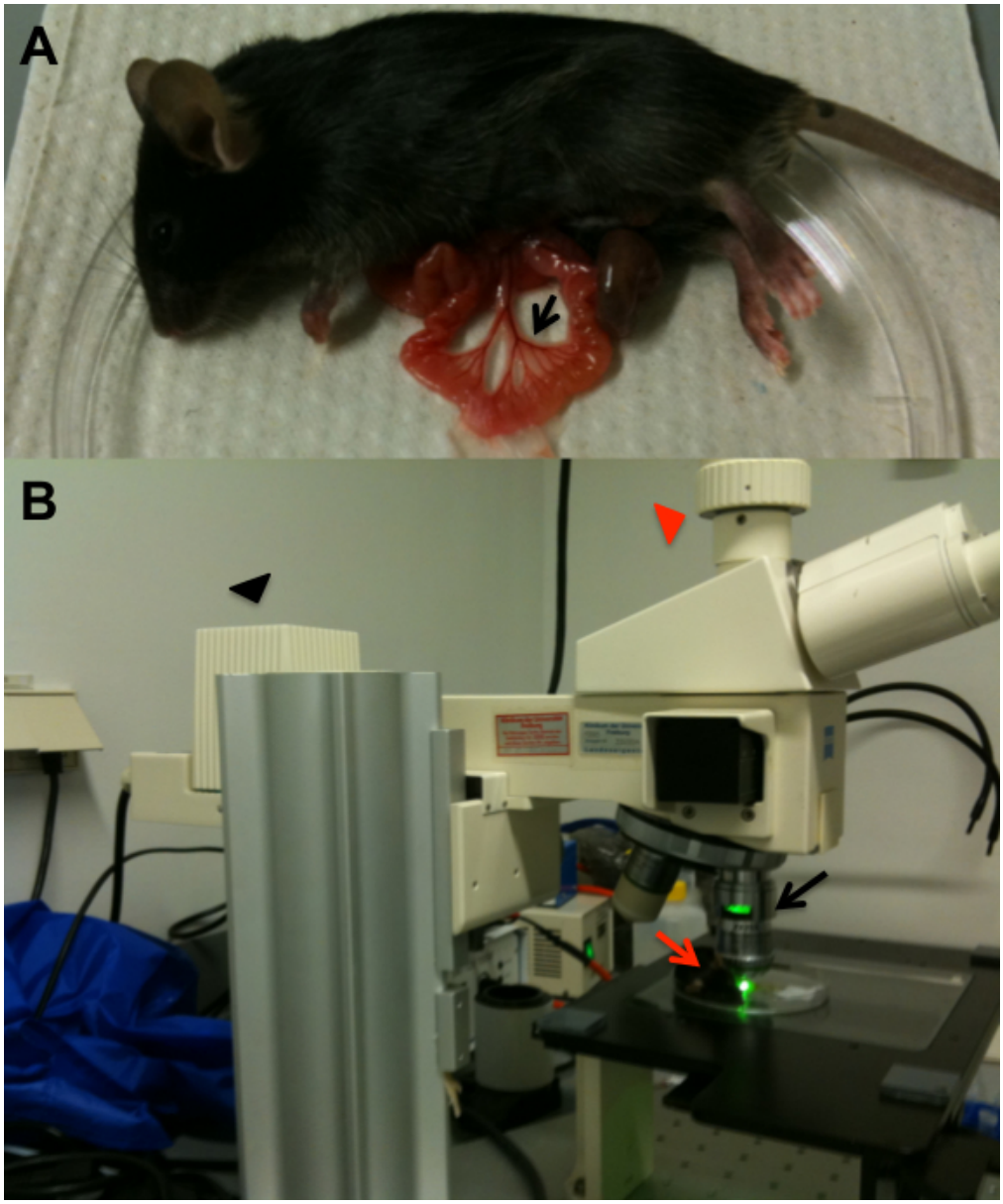

Figure 1. Overview of the Experimental Settings. (**A**) An anaesthetized mouse with exteriorized ileum-loop and its mesentery vessels (black arrow). (**B**) Intravital microscope with placed mouse (red arrow) underneath the objective (black arrow). Light source (black arrowhead) and camera (red arrowhead) are marked. Please click here to view a larger version of this figure.

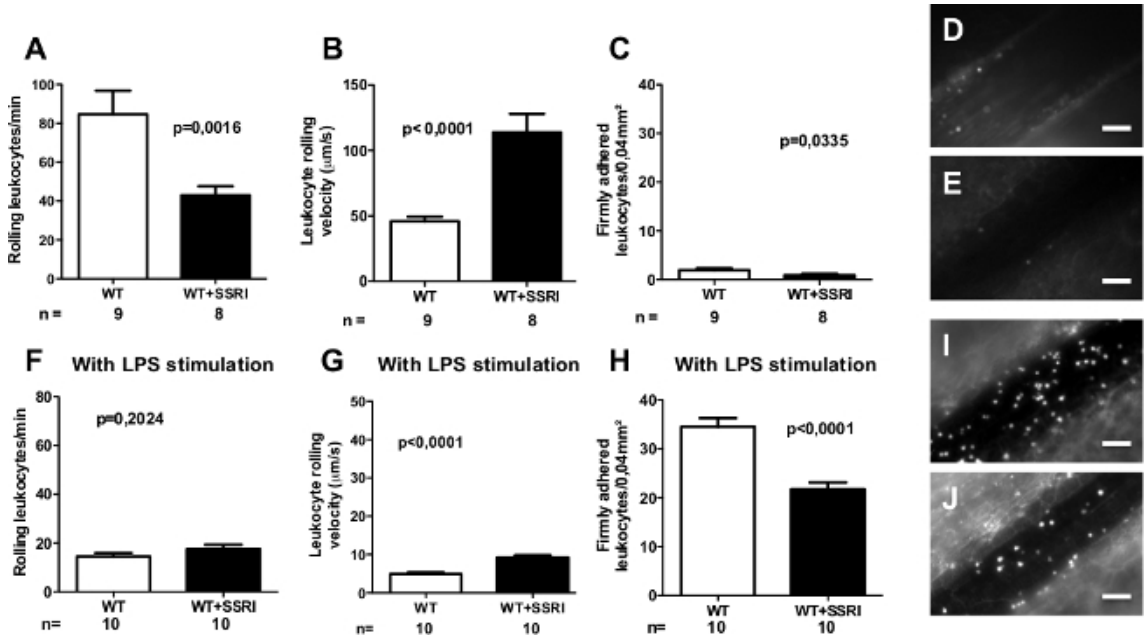

Figure 2. Intravital Microscopy After 3-weeks Administration of Fluoxetine to Wildtype Mice Either With or Without LPS Challenge. Modified from[7] (**A**) Number of rolling leukocytes without LPS-stimulation. (**B**) Leukocyte velocity without LPS stimulation. (**C**) Number of firmly adherent leukocytes without LPS stimulation. (**D**) Example of a vessel of an untreated WT mouse without LPS challenge. (**E**) Example of a vessel of a fluoxetine-treated mouse without LPS challenge. (**F**) Number of rolling leukocytes after LPS-stimulation. (**G**) Leukocyte velocity after LPS stimulation. (**H**) Number of firmly adherent leukocytes after LPS stimulation. (**I**) Example of a vessel of an untreated WT mouse after LPS challenge. (**J**) Example of a vessel of a fluoxetine-treated mouse after LPS challenge. Scale bar = 100 µm. Please click here to view a larger version of this figure.

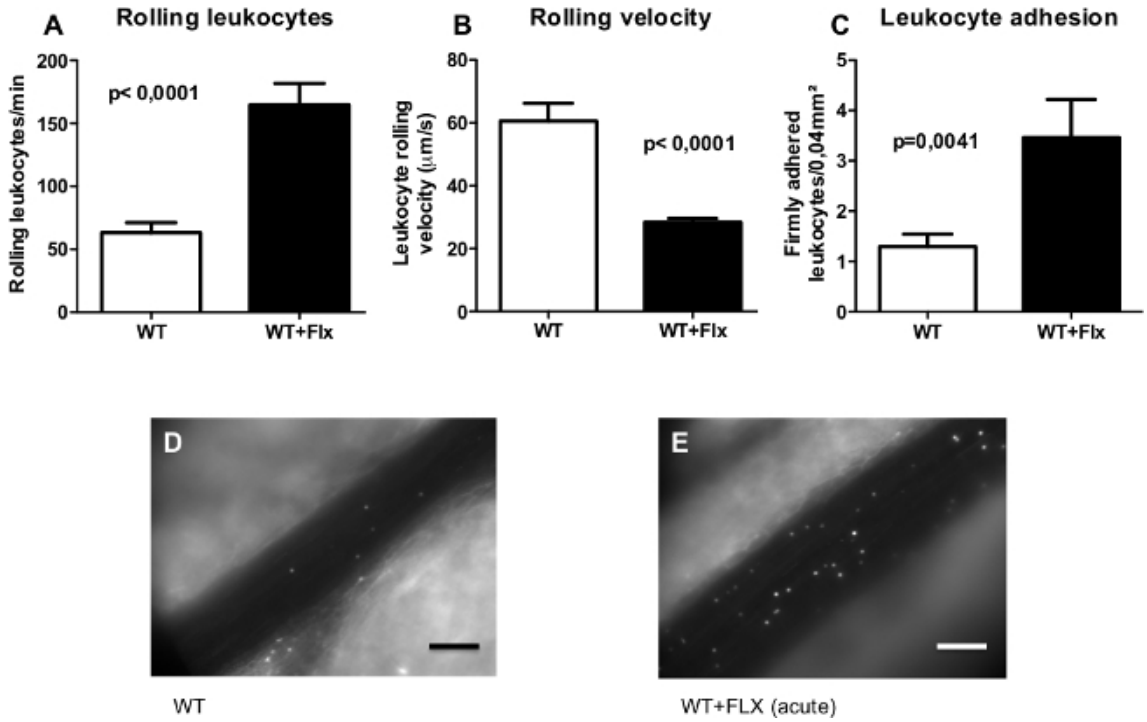

Figure 3: Intravital Microscopy After Acute Fluoxetine Challenge 2 hr Prior to Surgery Without LPS Challenge. Modified from[8] A) Number of rolling leukocytes without LPS-stimulation. B) Leukocyte velocity without LPS stimulation. C) Number of firmly adherent leukocytes without LPS stimulation. D) Example of a vessel of an untreated WT mouse without LPS challenge. E) Example of a vessel of an acute fluoxetine-treated mouse without LPS challenge. Scale bar = 100 µm. Please click here to view a larger version of this figure.

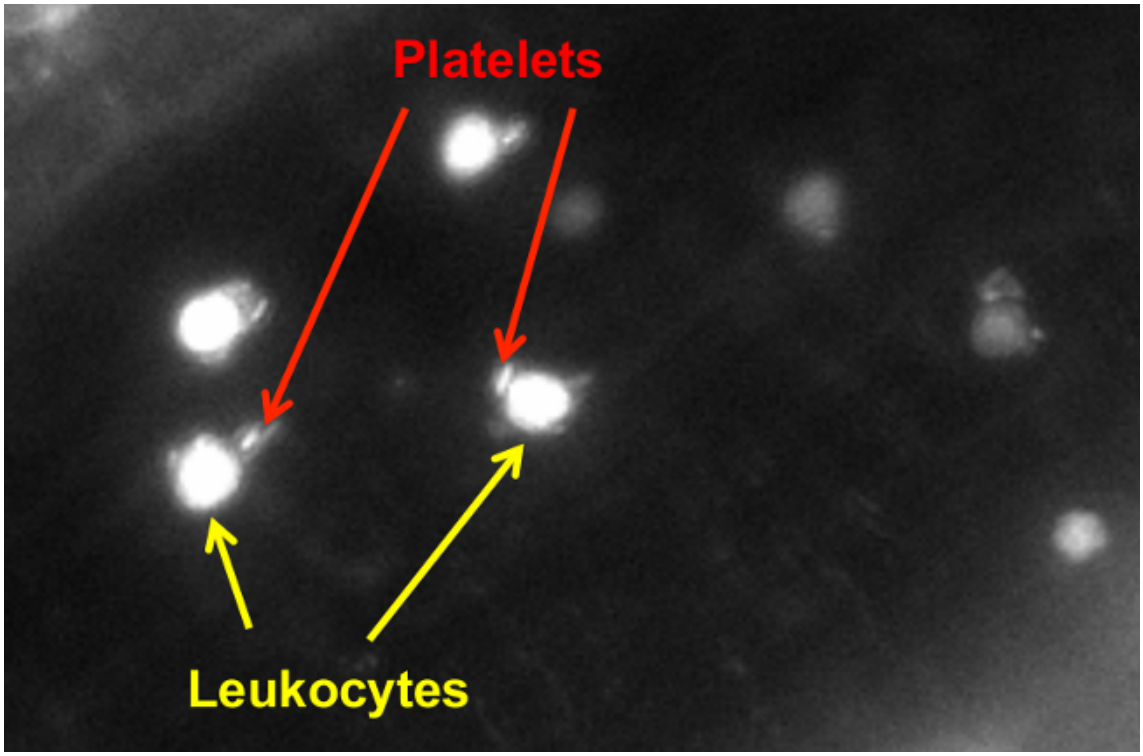

Figure 4: Platelet-leukocyte Interactions *in vivo*. Please click here to view a larger version of this figure.

Representative picture of platelet-leukocyte interactions in mice *in vivo* during lipopolysaccharide induced peritonitis in veins. Reprint from[11].

Discussion

Herein we describe the preparation and performance of intravital microscopy of mesentery veins of mice *in vivo*. This method gives us the opportunity to observe leukocyte-endothelial and platelet-endothelial interactions[11] directly in the living organism.

As an early response to inflammation the endothelium gets activated and interacts with leukocytes and platelets resulting in rolling, adhesion and transmigration[12]. But leukocytes also interact with platelets forming so called, platelet-leukocyte complexes, predominantly platelet-neutrophil complexes (PNCs) and platelet-monocyte complexes (PMCs)[13,14].These interactions can be observed and quantified in this method.

Additionally this technique allows investigating endothelium activation with markers like VCAM-1 or ICAM-1 (*e.g.,* after platelet/leukocyte interactions)[15].

The physical stimulus of the procedure itself causes a certain degree of endothelial activation, which leads to visible rolling of leukocytes (but almost no slow rolling and no firm adhesion). This implies that the examiner needs to handle the mesentery with great care. If there is too much activation caused by crude handling it will result in elevated numbers of rolling and adherent leukocytes. To reach a reproducible number of 30 - 80 rolling leukocytes per minute in wild-type mice the examiner has to be trained.

Blockage of P-selectin/PSGL-interaction could serve as a control to confirm the interaction of leukocytes with endothelium. It rules out that leukocytes are passively “attached” to the endothelium, a scenario that may occur if the blood flow is strongly reduced.

Mesentery vessel diameter ranges about 200 - 300 µm. Hence, this method is not suitable to assess microcirculation in capillaries, but focuses on leukocyte-endothelial interactions in small, inflamed veins, the primary place for transmigration of leukocytes. Compared to other intra-vital microscopy methods such as the cremaster model, this method is easier to learn and can be performed in a shorter time period.

One of the most important limitations of this method is that the mice need to be young without much mesentery fat to have a good view into the vessels, so that this method cannot be performed after long feeding or treatment studies.

Once the examiner is trained, different inflammatory conditions can be examined with this method, such as application of LPS, thioglycollate, TNFα or histamine. More specific imaging of particular blood cells can be achieved by using targeted fluorescently labeled molecules. Overall, this method is easy to learn and a quick way to examine leukocyte-endothelial and platelet-leukocyte interactions in a living organism.

Disclosures

The authors have nothing to disclose.

Acknowledgements

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